

CD47 Signals T Cell Death¹

Rolf D. Pettersen,^{2*} Kjetil Hestdal,* Mette Kløystad Olafsen,* Sverre O. Lie,[†] and Frederik P. Lindberg[‡]

Activation-induced death of T cells regulates immune responses and is considered to involve apoptosis induced by ligation of Fas and TNF receptors. The role of other receptors in signaling T cell death is less clear. In this study we demonstrate that activation of specific epitopes on the Ig variable domain of CD47 rapidly induces apoptosis of T cells. A new mAb, Ad22, to this site induces apoptosis of Jurkat cells and CD3ε-stimulated PBMC, as determined by morphological changes, phosphatidylserine exposure on the cell surface, uptake of propidium iodide, and true counts by flow cytometry. In contrast, apoptosis was not observed following culture with anti-CD47 mAbs 2D3 or B6H12 directed to a distant or closely adjacent region, respectively. CD47-mediated cell death was independent of CD3, CD4, CD45, or p56^{lck} involvement as demonstrated by studies with variant Jurkat cell lines deficient in these signaling pathways. However, coligation of CD3ε and CD47 enhanced phosphatidylserine externalization on Jurkat cells with functional CD3. Furthermore, normal T cells required preactivation to respond with CD47-induced apoptosis. CD47-mediated cell death appeared to proceed independent of Fas or TNF receptor signaling and did not involve characteristic DNA fragmentation or requirement for IL-1β-converting enzyme-like proteases or CPP32. Taken together, our data demonstrate that under appropriate conditions, CD47 activation results in very rapid T cell death, apparently mediated by a novel apoptotic pathway. Thus, CD47 may be critically involved in controlling the fate of activated T cells. *The Journal of Immunology*, 1999, 162: 7031-7040.

T cell responses to TCR-peptide/MHC interactions critically depend on the involvement of accessory molecules. Functional T cell activation requires a second signal provided by interactions of costimulatory molecules with distinct differentiation Ags presented by APC (1-4). In this context, coligation of the TCR by Ag and of CD28 by the B7 family of counterligands expressed on APC provides signaling for initiation and maintenance of T cell immune responses and protection from cell death during induction of target cell lysis (5-11). Experiments with CD28-deficient mice suggest that also other molecules may provide costimulation, as these mice generated CTLs in response to lymphocytic choriomeningitis virus (12). In contrast, TCR engagement in the absence of proper coactivation may induce T cell anergy and deletion via apoptotic cell death (13-15).

Negative regulators of T cell activation and molecules inducing cell death balance the level of mature T cells. Thus, mature peripheral T cells may respond with activation-induced apoptosis (AICD)³ following religation of the TCR/CD3 complex (16, 17). The Fas Ag is recognized as one of the major cell surface Ags involved in regulating the level of effector T cells by AICD (18-20). A critical role for Fas in the regulation of immune homeostasis

is strongly supported by the development of generalized lymphoproliferative disorders in *lpr* and *gld* mice with mutations in the genes for Fas and Fas ligand (FasL), respectively (21, 22). However, although Fas expression is rapidly induced on activated T cells, they apparently require several days after initial Fas expression to develop susceptibility to CD95-mediated death signaling (18, 23, 24). Recent experiments demonstrate that activation of TNFRI/p55 and TNFRII/p75 also may contribute to deletion of mature peripheral T cells (25-27). Interestingly, CD4⁺ and CD8⁺ T cells have apparently different sensitivity to TNF- and FasL-mediated apoptosis (26).

The cytotoxic T lymphocyte-associated Ag CTLA-4 is further implicated as an important regulator of T cell responses. The Ag, which is expressed on both activated CD4⁺ and CD8⁺ T cells (28), is related to the CD28 molecule and also binds the B7 family members CD80 and CD86 (7). Several studies suggest a major role for CTLA-4 in controlling proliferation and even mediating apoptosis (29-32). Thus, mice with a targeted mutation of CTLA-4 develop a spontaneous and fatal lymphoproliferative disease (32). Furthermore, studies of CTLA-4^{-/-} mice shows that CTLA-4 Ig treatment prevents fatal lymphoproliferation and suggest a role for CTLA-4 in the induction and maintenance of tolerance (33). In contrast, CTLA-4 has also been implicated in coactivation (28). Notably, CD80 costimulation of CTLA-4 promotes clonal expansion of CD28-deficient T cells (34). In addition, B7-CTLA-4 interaction enhances the production of antitumor CTL and resistance to tumor challenge (35). Thus, the biologic role for CTLA-4 has not been clearly established (36).

Other signaling pathways may also influence T cell proliferation and survival, and recent reports suggest a role for CD47 in costimulation. The 50-kDa Ig gene superfamily member CD47 is expressed on most human cells, including immature and mature T cells. Structurally, CD47 is composed of an extracellular IgV domain, five transmembrane domains, and a short cytoplasmic domain (37, 38). As the molecule physically and functionally associates with β₂ integrins on several cell types, CD47 is frequently

Departments of ¹Pediatric Research and ²Pediatrics, The National Hospital, Oslo, Norway; and ³Department of Infectious Diseases, Washington University School of Medicine, St. Louis, MO 63110

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² Address correspondence and reprint requests to Dr. Rolf D. Pettersen, Department of Pediatric Research, Rikshospitalet, The National Hospital, N-0027 Oslo, Norway. E-mail address: rolf.pettersen@kildimed.uio.no

³ Abbreviations used in this paper: AICD, activation-induced apoptosis; FasL, Fas ligand; TNFRI, TNF receptor type I; TNFRII, TNF receptor type II; IgV, Ig variable; IAP, integrin-associated protein; ICE, IL-1β-converting enzyme; PS, phosphatidylserine; PI, propidium iodide.

referred to as integrin-associated protein (IAP) (39). Specifically, recent studies demonstrate that coligation of CD3 and IAP (or CD47) may mobilize p56^{lck}, induce IL-2 production, and initiate T cell proliferation independent of CD28 signaling (40–42). However, high concentrations of anti-CD47 mAbs may also interfere with allogeneic MLRs (42), thereby indicating a possible dual role for the Ig superfamily member in regulating T cell responses.

In this study we demonstrate that ligation of distinct epitopes on CD47 rapidly induces the death of activated T cells, apparently independently of Fas and TNFR signaling. Furthermore, our work suggests that CD47 may be involved in the regulation of immune responses by activation of a novel death pathway. Thus, expression of the proper CD47 ligand(s) on APC may represent an efficient strategy to clear preactivated T cells.

Materials and Methods

mAbs and chemicals

The OKT3 hybridoma (anti-CD3 ϵ , IgG2a) was obtained from American Type Culture Collection (Manassas, VA). Apoptosis-inducing anti-CD95 (CH11, IgM) was purchased from Immunotech (Marseille Cedex, France). Anti-CD47 mAbs 2D3 (IgG1), B6H12 (IgG1), and 1F7 (IgG1) have been described previously (37, 39). Murine IgG1 (MOPC-21) control and recombinant human TNF- α and TNF- β were purchased from Sigma (St. Louis, MO). Murine IgM control (TEPC 183) was obtained from Biospecific (Emeryville, CA). Anti-CD120a (TNFR/p55; IgG1-PE) and anti-CD120b (TNFR/p75; IgG1-PE) were obtained from Boehringer Ingelheim (Heidelberg, Germany). Anti-CD4 (IgG1-FITC), anti-CD8 (IgG1-PE), IgG1-FITC, and IgG1-PE controls were purchased from Becton Dickinson (San Jose, CA). Streptavidin-FITC was obtained from Dako (Glostrup, Denmark), and streptavidin-PE was obtained from Southern Biotechnology Associates (Birmingham, AL). Recombinant human TNFR/Fc and Fas-Fc chimera were obtained from R & D Systems (Abingdon, U.K.). ICE inhibitor II (Ac-Tyr-Val-Ala-Asp-chloromethyl ketone; Ac-YVAD-CMK) was purchased from Calbiochem (La Jolla, CA). ICE inhibitor Z-Val-Ala-DL-Asp-fluoromethyl ketone (Z-VAD-FMK) and CPP32 inhibitor Ac-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were purchased from Bachem Biochemicals (Bubendorf, Switzerland). Cytochalasin D was obtained from Calbiochem (La Jolla, CA).

mAb production, cell culture, and transfectants

mAb Ad22 (IgG1 κ) was raised against the human T cell line PFT-285 (43) as previously described (44). Human cells were cultured in RPMI 1640 supplemented with 10% FCS, 1.5 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. CD47-deficient human ovarian carcinoma OV10 cells and CD47-expressing OV10 transfectants have been described previously (45). The human promonocytic cell line U937, the human T cell line Jurkat E6, and Jurkat derivatives JRT3-T3.5 (TCR $\alpha\beta$ /CD3 deficient), J.CaM1.6 (defective in p56^{lck} kinase expression), J45.01 (CD45 deficient), and D1.1 (CD4 negative) were obtained from American Type Culture Collection. Cell lines were subcultured every 2–3 days to maintain cell densities between 1×10^5 and 1×10^6 cells/ml. Experiments were performed on 5×10^5 cells/ml cultured in 24-well cell culture cluster plates (3524, Costar, Cambridge, MA) unless otherwise indicated. Human PBMC were isolated by Lymphoprep (Nycoprep Pharmacia, Oslo, Norway) centrifugation. All cultures were grown at 37°C in a humidified 5% CO₂ atmosphere.

Flow cytometry

Ag expression was determined with flow cytometry as previously described (46). Cells were finally washed twice in PBS and fixed with 1% paraformaldehyde in PBS. Samples were analyzed using a FACSort (Becton Dickinson), and data were collected for 10,000 cells.

Flow cytometric determination of apoptosis and cell death

Apoptosis was determined by monitoring changes in cell size and granularity by flow cytometry and assessment of phosphatidylserine (PS) exposure by annexin V-FUOS (Boehringer Mannheim, Mannheim, Germany) binding according to the manufacturer's instructions. DNA fragmentation was determined with the TUNEL assay kit from Boehringer Mannheim. Cell membrane permeability was assessed by determining uptake of the DNA-binding fluorescent dye propidium iodide (PI; 2.5 μ g/ml) after incubation for 10 min. In each case data were collected for 10,000 cells. TruCount tubes (Becton Dickinson) containing fluorescent-dyed microbeads were used to assess relative cell numbers.

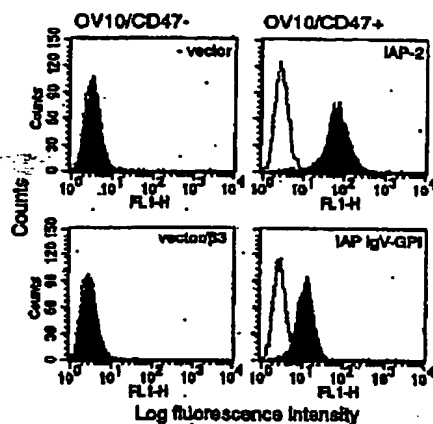


FIGURE 1. mAb Ad22 reacts with epitopes on the IgV domain of CD47. Domain specificity for Ad22 binding was determined using OV10 transfectants expressing intact CD47 (IAP-2) and CD47 IgV domain fused to human decay-accelerating factor-glycosylphosphatidylinositol (IAP IgV-GPI) (43). OV10 and OV10 cells transfected with vector and a β_2 integrin expression plasmid (43) were included as negative controls. Ag expression (filled histograms) was determined using biotinylated Ad22 and streptavidin-FITC followed by analysis with flow cytometry. Open histograms represent staining with biotinylated control mAb. Both Ad22 and 2D3 showed reduced reactivity with IAP IgV-GPI compared with IAP-2 transfectants (data not shown).

Proliferation assays with immobilized mAb

Proliferation assays were performed in flat-bottom 96-microwell plates (Costar) coated with mAb (1, 5, or 10 μ g/ml) in PBS (100 μ l/well) for 20–24 h at 4°C. Before the start of culture, the mAb solutions were aspirated, and each well was washed three times with 250 μ l of PBS. Jurkat E6 cells (5×10^5 cells/ml) were cultured for 24 h with addition of 1 μ Ci of [³H]thymidine (Amersham, Aylesbury, U.K.) to each well for the last 6 h. Cells were harvested onto glass-fiber filters with a Skatron harvester (Skatron, Lierakogen, Norway), and [³H]thymidine incorporation was determined using a Betaplate scintillation counter (Wallac Oy, Turku, Finland).

Results

T cell death is induced by activation of distinct IgV CD47 epitopes

The functional role(s) of CD47 on T cells is not clear. However, recent studies suggest that CD47 may serve as a costimulatory molecule in T cell activation (40–42). We produced a new mAb (Ad22, IgG1 κ) reacting with the IgV domain of CD47 (Fig. 1) in proximity to epitopes defined by mAb B6H12 and 1F7 (Fig. 2) and addressed the functional role of this region on human T cells. Jurkat cells were used as a model system, as these cells can respond with coactivation or apoptosis following engagement of different integral membrane proteins (40–42, 47–49). Surprisingly, microscopic examinations and assessments with flow cytometry revealed that Jurkat E6 cells incubated with Ad22 aggregated and showed morphological characteristics of apoptotic cells (data not shown). To further determine whether apoptosis could be signaled by activation of CD47, we examined the impact of IgG1 κ anti-CD47 mAbs Ad22, B6H12, 1F7 (directed to adjacent epitopes), and 2D3 (to a distant region) using flow cytometry.

During the early stages of apoptosis PS translocates from the interior to the exterior part of the plasma membrane and becomes exposed at the apoptotic cells (47). Annexin V binds with high affinity to PS and can therefore be used to identify cells in all stages of programmed cell death (47). PI only stains late apoptotic/dead cells due to a compromised cell membrane. Thus, staining

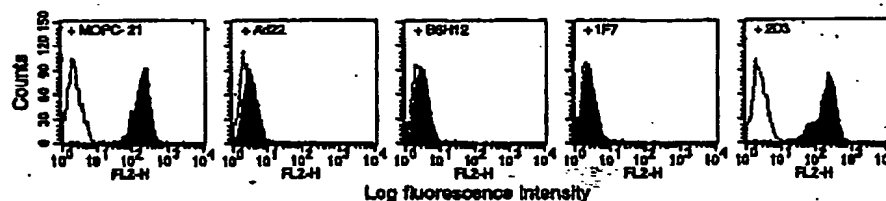


FIGURE 2. mAb Ad22, B6H12, and 1F7 bind overlapping or adjacent epitopes on the IgV domain of CD47. Competitive binding assays were used to determine the Ad22 binding region on CD47 relative to the sites recognized by mAbs B6H12, 1F7, and 2D3. PFL-285 cells (1×10^6) were preincubated for 30 min on ice with 50 μ g of mAb, as indicated, followed by Ad22-biotin (0.25 μ g) and streptavidin-PE addition. Ad22 binding (filled histograms) was determined with flow cytometry. Open histograms represent staining with isotype control IgG-biotin.

with both annexin V-FLUOS and PI will allow for distinguishing between early/intermediate apoptotic and late apoptotic/dead cells when the cells are analyzed with two-color flow cytometry (FL1 vs FL2). To assess the impact of CD47 engagement, Jurkat E6 cells were cultured with anti-CD47 mAbs and examined for annexin V-FLUOS binding and uptake of PI with flow cytometry. These studies clearly demonstrated that Ad22 rapidly induced a marked increase in both apoptotic (annexin V positive/PI negative) and dead (annexin V positive/PI positive) cells compared with control cultures (Fig. 3A and data not shown) even at low mAb concentrations (Fig. 3B). The response was readily observable within 30 min, and the effect, as determined by annexin V-FLUOS binding, reached a maximum within the first 3 h (Fig. 3C and data not shown). Furthermore, mAb 1F7 also induced PS exposure and uptake of PI (Fig. 3, A and B). In contrast, no significant impact on PS exposure or uptake of PI was observed with control mAb MOPC-21 or the anti-CD47 mAbs B6H12 or 2D3 after 1, 3, or 5 h of cell culture (Fig. 3, A and B, and data not shown). Thus, CD47 can signal apoptotic T cell death, and induction of a death response apparently requires ligation of distinct epitopes on the IgV domain of CD47.

Role of functional TCR/CD3, CD4, CD45, and p56^{lck} signaling in CD47-mediated apoptosis

AICD of T cells plays a critical role in the regulation of immune responses. During T cell activation, TCR signals are modulated by the tyrosine kinase p56^{lck}, which associates with the cytoplasmic domain of CD4 and CD8 (50–52). CD45 expression is also required for p56^{lck} activation and TCR signaling (50, 53, 54). Furthermore, recent studies demonstrate that ligation of CD4 and CD45 can induce elimination of T cells by apoptosis (48, 55–58), and that AICD of Jurkat T cells requires functional p56^{lck} (59, 60). Thus, as Ad22 rapidly induced death of Jurkat T cells, we asked whether CD47-induced apoptosis was related to AICD of T cells and/or required functional CD3, CD4, CD45, or p56^{lck}. To address these issues, Ad22 responses were assessed using established variant Jurkat T cell lines selected for deficient expression of TCR/CD3 (clone J.RT3-T3.5), CD4 (clone D1.1), CD45 (clone J45.01), or deficient p56^{lck} signaling (clone J.CaM1.6). As shown in Fig. 4, these experiments clearly demonstrate that Ad22 rapidly induced apoptosis of all the variant Jurkat clones as determined with annexin V binding and flow cytometry. In contrast, no cell death was observed with control mAb MOPC-21 or anti-CD47 mAb 2D3 or B6H12 after 1 or 3 h of culture (Fig. 4 and data not shown). Thus, CD47-induced apoptosis apparently proceeds independent of the signaling pathways required to initiate AICD of T cells.

Although CD47-induced apoptosis apparently was independent of signaling required to initiate AICD of T cells, we had not excluded the possible involvement of CD47 as a coactivator in TCR/CD3-induced apoptosis. Thus, to approach these possibilities,

Jurkat E6 cells were cultured with Ad22, 2D23, or control mAb MOPC-21 with or without the mitogenic anti-CD3 mAb OKT3 and examined for PS exposure and uptake of PI with flow cytometry. The TCR/CD3-deficient Jurkat clone J.RT3-T3.5 was included as a negative control. Interestingly, these assessments showed a profound increase in annexin V binding on Jurkat E6 cells treated with Ad22 and OKT3 for 1 or 3 h compared with that on cells only incubated with Ad22 (Fig. 5 and data not shown). In contrast, no significant induction of annexin V binding was observed with a combination of OKT3 and MOPC-21 or OKT3 and 2D3 (Fig. 5 and data not shown). Furthermore, OKT3 had no impact on PS exposure in the control cell line J.RT3-T3.5 (data not shown). Taken together, CD47-induced apoptosis is apparently independent of early TCR-related signaling, but coactivation of these pathways enhances the apoptotic response.

Unrestricted CD47 domains and an intact cytoskeleton are required for death signaling

T cell death was observed with even low concentrations of soluble Ad22, and soluble anti-CD47 mAb 1F7 also induced PS externalization (Fig. 3). To further confirm and understand the distinct requirements for CD47 engagement in T cell death signaling we first examined the responses of Jurkat E6 to Ad22 following preincubation with excess anti-CD47 mAb B6H12 or 2D3. Similar experiments with control mAb MOPC-21 were included. Based on assessments of annexin V binding with flow cytometry, we found that mAb B6H12, which interferes with Ad22 binding to CD47 (Fig. 2), efficiently blocked Ad22-imposed death responses (Fig. 6A). Notably, preincubation with anti-CD47 mAb 2D3, which had no impact on Ad22-CD47 interactions (Fig. 2), also profoundly reduced death responses to Ad22 (Fig. 6A). In contrast, MOPC-21 had no influence on Ad22-induced death of Jurkat E6 cells (Fig. 6A). Thus, restrictions in both Ad22-CD47 interactions and Ag structure flexibility interfere with death signaling. Furthermore, we examined the effect of immobilized Ad22, 2D3, and control mAb MOPC-21 on Jurkat E6 cells cultured for 24 h. These experiments were based on assessments of [³H]thymidine incorporation, as immobilized anti-CD47 mAbs induced cell adherence to the plastic surface of the microtiter wells (data not shown). At all concentrations examined (1, 5, and 10 μ g/ml) we found that immobilized mAb Ad22, 2D3, or MOPC-21 had no influence on Jurkat cell proliferation (data not shown). Thus, the mode of Ad22 presentation is critical with respect to functional responses.

These data suggested distinct requirements related to CD47 conformation and mobility for death signaling. As the actin cytoskeleton may be involved in receptor reorganization and signaling (61), we further addressed the requirement for an intact actin cytoskeleton in CD47-mediated death signaling. In these experiments we examined the effect of Ad22 on Jurkat E6 cells with or without pretreatment with the actin polymerization inhibitor cytochalasin

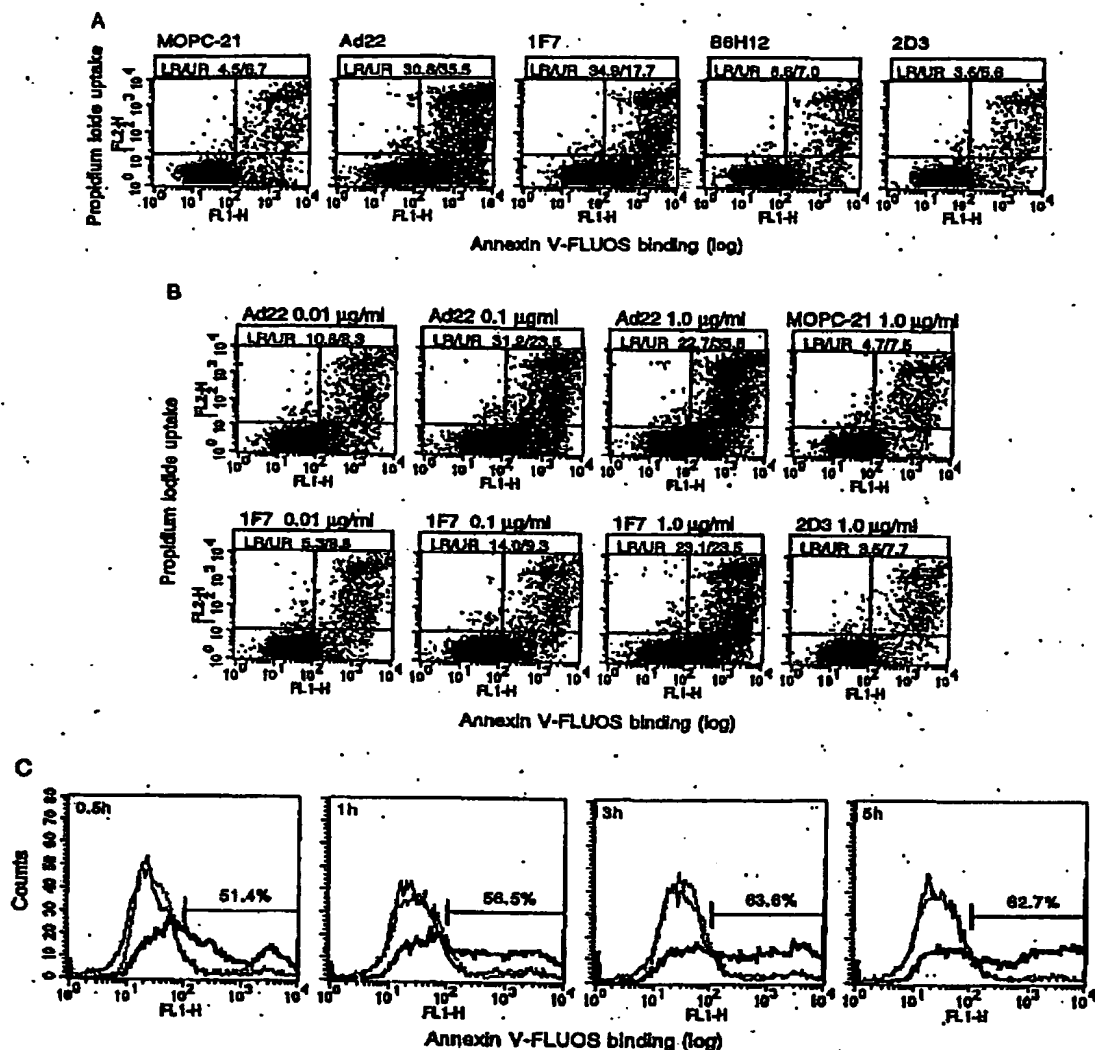


FIGURE 3. Anti-CD47 mAbs Ad22 and 1F7 induce externalization of PS and uptake of PI. Jurkat E6 cells were cultured with anti-CD47 mAbs Ad22, 1F7, B6H12, 2D3, or control mAb MOPC-21 as indicated and examined for annexin V-FLUOS binding and uptake of PI with flow cytometry. The numbers at the top of the dot plots indicate the percentage of cells in the lower right (LR) and upper right (UR) regions, respectively. **A**, Annexin V-FLUOS and PI staining induced following 3-h incubation with 1 $\mu\text{g/ml}$ mAb. **B**, Dose responses of Ad22 and 1F7 after 3 h. Control cultures were incubated with 1 $\mu\text{g/ml}$ MOPC-21 or 2D3. **C**, Kinetics of PS exposure induced by 1 $\mu\text{g/ml}$ Ad22. The percentage of annexin V-positive cells following Ad22 incubation (solid, bold lines) is indicated. The other histograms represent annexin V-FLUOS staining following MOPC-21 (solid lines) and 2D3 (dotted lines) incubation.

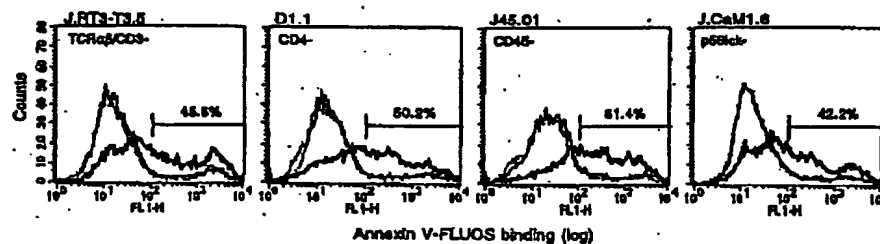


FIGURE 4. CD47 signals apoptosis independent of functional CD3, CD4, CD45, or p56^{lck}. Variant Jurkat cells deficient in TCR $\alpha\beta$ /CD3 (J.RT3-T3.5), CD4 (D1.1), CD45 (J45.01), or p56^{lck} (J.CaM1.6) were cultured with anti-CD47 mAbs Ad22, 2D3, or control mAb MOPC-21 (1 $\mu\text{g/ml}$) for 1 h and examined for annexin V-FLUOS binding with flow cytometry. The percentage of annexin V-positive cells following Ad22 incubation (solid, bold lines) is indicated. The other histograms represent annexin V-FLUOS staining following MOPC-21 (solid lines) and 2D3 (dotted lines) incubation.

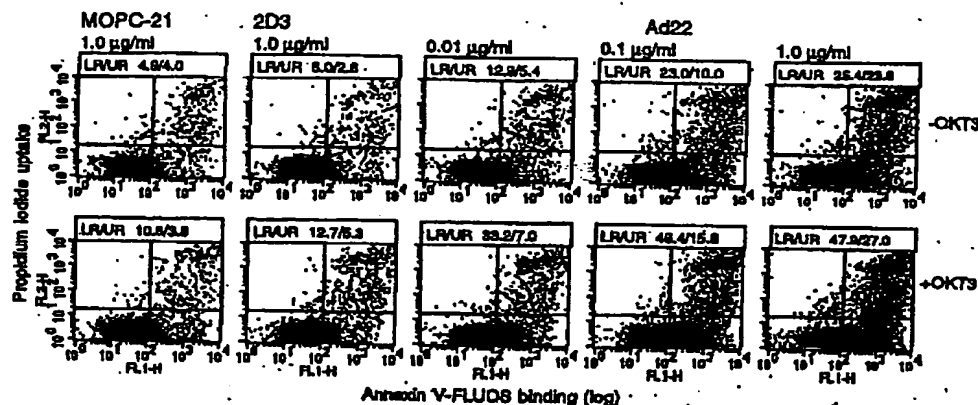


FIGURE 5. Coligation of CD3 ϵ enhances PS externalization induced by mAb Ad22. Jurkat E6 cells were cultured with Ad22, 2D3, or control mAb MOPC-21 with or without OKT3 (1 μ g/ml) for 1 h as indicated and examined for annexin V-FLUOS binding and uptake of PI with flow cytometry. The numbers at the top indicate the percentage of cells in the lower right (LR) and upper right (UR) regions, respectively.

D. Control experiments with MOPC-21 were also included. Based on assessments of annexin V binding with flow cytometry we found that interference with actin polymerization profoundly reduced death responses following CD47 engagement (Fig. 6B). Taken together, death signaling by CD47 apparently requires an unrestricted and flexible Ag structure and involvement of the actin cytoskeleton.

CD47-induced apoptosis is independent of TNFR and Fas signaling

TCR-induced apoptosis can result from TNFR/p55, TNFR/p75, and Fas activation (25–27). Thus, to assess whether these TNFR superfamily members and related signaling pathways were involved in CD47-mediated apoptosis, we first examined the impact of recombinant human TNF- α and TNF- β (from 10 pg/ml to 1.0 μ g/ml in increments of 10-fold) on Jurkat E6 cells in short term cultures. The TNF-sensitive human promonocytic cell line U937 was included as a positive control to confirm cytokine activity.

These studies revealed that the cytokines had no effect on PS exposure on Jurkat E6 cells following 3- and 5-h cultures (data not shown). In comparison, control cultures with Ad22 responded with extensive PS exposure as previously observed (data not shown). Cultures with U937 confirmed cytokine activity and revealed significant death responses with concentrations as low as 10 pg/ml for TNF- α and 100 pg/ml for TNF- β after 18 h (data not shown). Flow cytometric assessments revealed low expression of CD120a and undetectable expression of CD120b on Jurkat E6, while both receptors were clearly expressed on U937 (data not shown). To further examine whether Ad22 imposed increased TNF sensitivity to T cells, we performed experiments to assess the impact of Ad22 on Jurkat E6 cells preincubated with soluble human recombinant TNFRI/Fc to block TNFR-TNF interactions or with control IgG (MOPC-21). Control cultures with U937 incubated with recombinant TNFRI/Fc or MOPC-21 and TNF- α or TNF- β (10 and 100 ng/ml) were also included. We found no interference of soluble human recombinant TNFRI/Fc with the ability of Ad22 to induce PS exposure on Jurkat E6 cells after 1 or 3 h of culture (Fig. 7). In contrast, TNFRI/Fc pretreatment of U937 cells profoundly interfered with TNF- α and TNF- β -induced cell death (Fig. 7 and data not shown). Thus, CD47-induced apoptosis apparently did not proceed by promoting TNF- α or TNF- β interactions with TNFRs and subsequent activation of the TNFR signaling pathways.

Jurkat E6 cells express CD95 and initiate programmed cell death following incubation with the anti-Fas mAb CH11 (47). To determine whether Fas activation was initiated by the Ad22 mAb, we first compared the kinetics of annexin V binding to Jurkat E6 cells following incubation with 1 μ g/ml Ad22 or CH11. These assessments showed that even after 6 h with CH11, the percentage of annexin V-positive cells had not reached the same level as that of Jurkat E6 cells incubated with Ad22 for 30 min (data not shown). Furthermore, we also determined the impact of Ad22 on Jurkat E6 cells preincubated with human recombinant Fas-Fc chimera to block Fas-FasL interactions or with control IgG (MOPC-21). Control cultures with CH11 were included. These assessments clearly revealed that whereas recombinant Fas-Fc abolished the effect of CH11 in 6-h cultures, blocking functional Fas-FasL interactions had no impact on the ability of Ad22 to induce PS exposure on Jurkat cells after 1 or 3 h (Fig. 8). Thus, CD47-mediated apoptosis apparently proceeds without Fas activation.

Apoptosis may proceed with or without DNA fragmentation. Thus, whereas Fas-activated cell death involves DNA cleavage

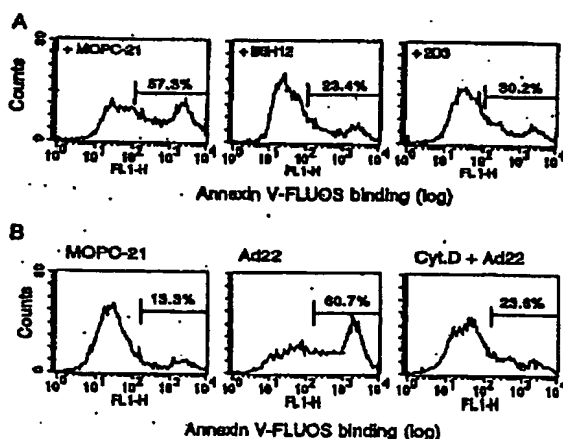
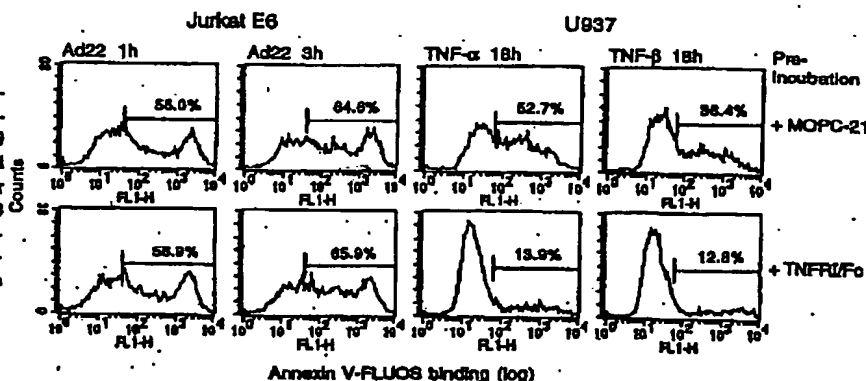


FIGURE 6. Ad22-induced cell death requires an unrestricted CD47 Ag and intact actin cytoskeleton. A, Jurkat E6 cells were preincubated with 1 μ g/ml MOPC-21, B6H12, or 2D3 for 15 min as indicated and further cultured with 0.1 μ g/ml Ad22 for 3 h. B, Jurkat E6 cells were cultured with 1 μ g/ml control mAb MOPC-21 or Ad22 with or without 15-min pretreatment with 20 μ M cytochalasin D for 3 h as indicated. Apoptosis was determined by examining annexin V-FLUOS binding with flow cytometry.

FIGURE 7. CD47 mediates T cell death independent of TNFR ligation. Cells were preincubated for 15 min with 1 μ g/ml control IgG (MOPC-21) or recombinant human TNFR/Fc and were further cultured with 1 μ g/ml Ad22 for 1 or 3 h (Jurkat E6) or with 10 ng/ml TNF- α or TNF- β for 18 h (U937) as indicated. Apoptosis was determined by examining annexin V-FLUOS binding with flow cytometry. Results are representative of two separate experiments.



into oligonucleosomes (47), CD45 ligation may induce apoptosis without observable DNA fragmentation (48). To further address how CD47-mediated apoptosis proceeds, Jurkat E6 cells were cultured with anti-CD47 mAbs Ad22 or 2D3 or control mAb MOPC-21 for 1, 2, or 3 h and examined for induction of DNA fragmentation with the TUNEL assay. Jurkat E6 cultures treated with the apoptosis-inducing Fas mAb CH11 for 6 h were included as positive controls. In these studies examinations with flow cytometry showed no characteristic DNA nicks following incubation with Ad22 or 2D3 (Fig. 9 and data not shown). Furthermore, as combined OKT3 and Ad22 treatment showed enhanced annexin V binding compared with Ad22 alone (Fig. 5), we similarly examined the impact of CD3 and CD47 costimulation with the TUNEL assay. These experiments revealed no significant increase in staining intensity in cultures with both OKT3 and Ad22 compared with that in cultures with Ad22 alone or in control cultures (Fig. 9 and data not shown). However, in contrast to Ad22, CH11 clearly induced DNA fragmentation (Fig. 9). Taken together, CD47-mediated apoptosis apparently proceeds independently of TNFRs and Fas involvement and does not involve DNA fragmentation.

Roles of ICE and CPP32

Activation of ICE- and CPP32-like proteases is required as a downstream event to accomplish programmed cell death following TNFRs and Fas ligation (62, 63). To address whether ICE and CPP32 activities also were involved in CD47-mediated apoptosis we assessed the impact of specific ICE and CPP32 peptide inhibitors on Ad22-induced responses of Jurkat E6 cells. Control cultures with CH11 (anti-Fas) were also included. Jurkat E6 cells

were preincubated for 1 h with the ICE inhibitors Ac-Tyr-Val-Ala-Asp-chloromethyl ketone and Z-Val-Ala-DL-Asp-fluoromethyl ketone or the CPP32 inhibitor Ac-Asp-Glu-Val-Asp-aldehyde and further cultured for 1 or 3 h with Ad22 or MOPC-21 or for 6 h with CH11 or isotype control mAb TEPC 183. Apoptosis was determined by assessment of annexin V-FLUOS binding with flow cytometry. These experiments showed that whereas Fas-mediated apoptosis was greatly suppressed by peptide inhibitors of ICE and CPP32, the apoptotic response induced by Ad22 was not significantly influenced by blocking ICE- or CPP32-like activity (Table I). Thus, CD47-mediated death of T cells does apparently not require ICE and CPP32 involvement.

CD47 signals death of CD3-stimulated, but not resting, normal T cells

Mature T cells can be eliminated by FasL- or TNF-induced apoptosis following reactivation of the TCR/CD3 complex (16, 17). Although CD47-mediated apoptosis seems to proceed independently of these death pathways, we asked whether resting or anti-CD3-stimulated normal T cells also responded with cell death when challenged with the Ad22 mAb. To address the role of CD47 signaling in T cells, we determined the impact of Ad22, 2D3, and control mAb MOPC-21 on unstimulated and OKT3-stimulated PBMC in short term cultures. In these experiments we used Tru-Count tubes containing a known number of fluorescent-dyed microbeads (counts) to determine relative numbers of lymphocytes following mAb treatment, with flow cytometry. The region representing living lymphocytes (R1) was defined, and the number of cells in R1 was determined following sampling of 2000 reference

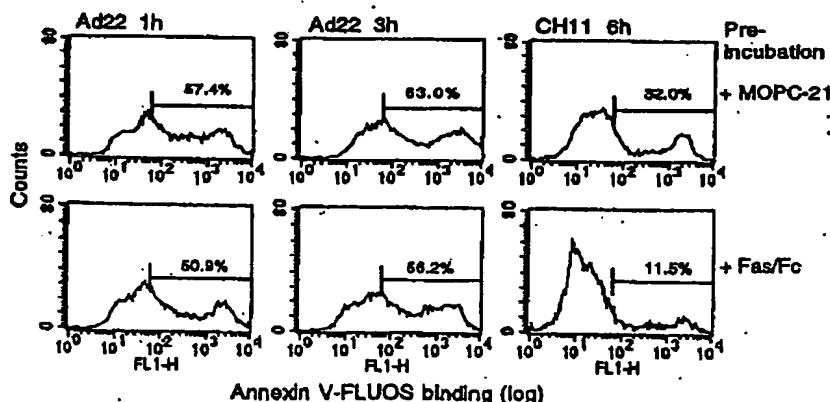


FIGURE 8. CD47 signals apoptosis independent of CD95-CD95L interactions. Jurkat E6 cells were preincubated for 30 min with 10 μ g/ml control IgG (MOPC-21) or recombinant human Fas-Fc chimera and further cultured with 1 μ g/ml Ad22 or 1 μ g/ml CH11 as indicated. Apoptosis was determined by examining annexin V-FLUOS binding with flow cytometry. Results are representative of two separate experiments.

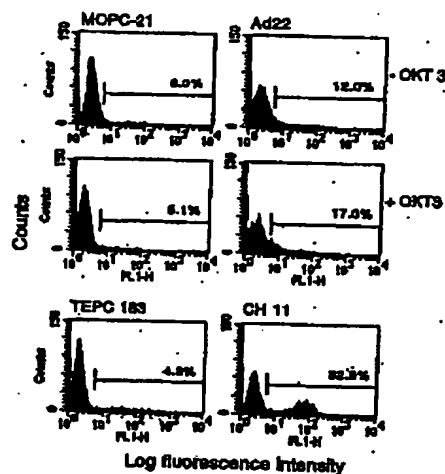


FIGURE 9. mAb Ad22-induced apoptosis proceeds without characteristic DNA fragmentation. Jurkat E6 cells were cultured with 1 μ g/ml Ad22 or MOPC-21 with or without OKT3 (1 μ g/ml) for 1 h or with anti-Pas mAb CH11 or isotype control mAb TEPC-183 (1 μ g/ml) for 6 h as indicated. DNA fragmentation was examined with the TUNEL assay. Incorporated biotin-16-dUTP was determined using streptavidin-FITC and flow cytometry. The percentage of stained cells is indicated. Results are representative of three separate experiments.

beads. With seven different donors, we found comparable numbers of lymphocytes in R1 when unstimulated PBMC were incubated with Ad22, 2D3, or isotype control mAb MOPC-21 for 24 h (Fig. 10 and data not shown). On the average, we found 91.1% (range, 78.0–101.1%) of the number of cells in R1 with Ad22 and 99.4% (range, 87.9–124.7%) of the number of cells in R1 with 2D3 compared with similar cultures with MOPC-21. In contrast, when cells were prestimulated for 18 h with OKT3 and then further incubated for 6 h with Ad22, we observed a marked decrease in the number of living lymphocytes compared with that in similar cultures with MOPC-21 or 2D3 (Fig. 10 and data not shown). Of 12 different samples of OKT3-stimulated PBMC, 11 responded to Ad22 with a reduction in the number of lymphocytes compared with control cultures with MOPC-21. On the average, these donors had 66.9% (range, 52.1–93.8%) of the number of lymphocytes compared with similar cultures with MOPC-21. With 2D3, we observed an average of 99.9% (range, 76.1–124%) of the number of cells in R1 compared with MOPC-21. Student's *t* test showed significant responses to Ad22 compared with MOPC-21 and 2D3 ($p < 0.001$; no data overlap) and no impact of 2D3 compared with MOPC-21

($p > 0.5$). Furthermore, both activated CD4⁺ and CD8⁺ cells appeared to be sensitive to Ad22 treatment (data not shown). Thus, activated, but not resting, normal T cells were sensitive to death signaling by CD47.

Discussion

T cell apoptosis plays an important role in controlling immune responses. In the present work we have demonstrated that engagement of distinct epitopes on CD47 rapidly signals T cell death independent of the TNFRI/p55, TNFRII/p75, or Fas signaling pathways. Thus, the data presented suggest that CD47 may represent a novel pathway for efficient clearance of activated T cells.

Recent studies implicate CD47 in diverse biological functions. In this context, a critical role for CD47 in regulating β_2 integrin-dependent migration and activation of PMN has been demonstrated using CD47-deficient mice (64). CD47 may also execute its functions independent of β_2 integrins. Thus, it has recently been shown that coligation of CD3 and CD47 on T cells, which do not express β_2 integrins, may enhance IL-2 production and T cell proliferation independent of CD28 signaling (40–42). Our studies apparently add induction of T cell apoptosis to the β_2 integrin-independent biologic repertoire of CD47.

The anti-CD47 mAbs B6H12 and 2D3 recognize distinct, non-overlapping regions of CD47 associated with functional β_2 integrin interactions and T cell activation, respectively (39, 40). Assessments with competitive mAb binding and flow cytometry revealed that Ad22, B6H12, and 1F7 recognize overlapping or adjacent epitopes on the extracellular IgV domain. Thus, these mAbs apparently define a unique region on CD47 associated with functional β_2 integrin association and induction of T cell apoptosis, respectively. However, assessments with Jurkat T cells revealed that Ad22 and 1F7 were unique among the anti-CD47 mAbs tested and were the only mAbs capable of inducing T cell death. Distinct death signaling by CD47 was directly confirmed, as both B6H12 and 2D3 interfered with Ad22 responses. The fact that 2D3, in contrast to B6H12, noncompetitively inhibits the death response to Ad22 suggests that a distinct conformation or conformational change in CD47 is required for death signaling. Furthermore, CD47 signaling appeared to be easily activated by proper stimulation, as low concentrations of Ad22 could initiate death responses, and moreover, the kinetics of PS exposure and PI uptake suggested a rapid transition through the apoptotic (annexin V⁺/PI⁺) stage.

T cell susceptibility to different forms of negative signaling depends on the differentiation stage. Developing thymocytes may

Table I. Effects of ICE and CPP32 peptide inhibitors on Ad22 induced annexin V binding^a

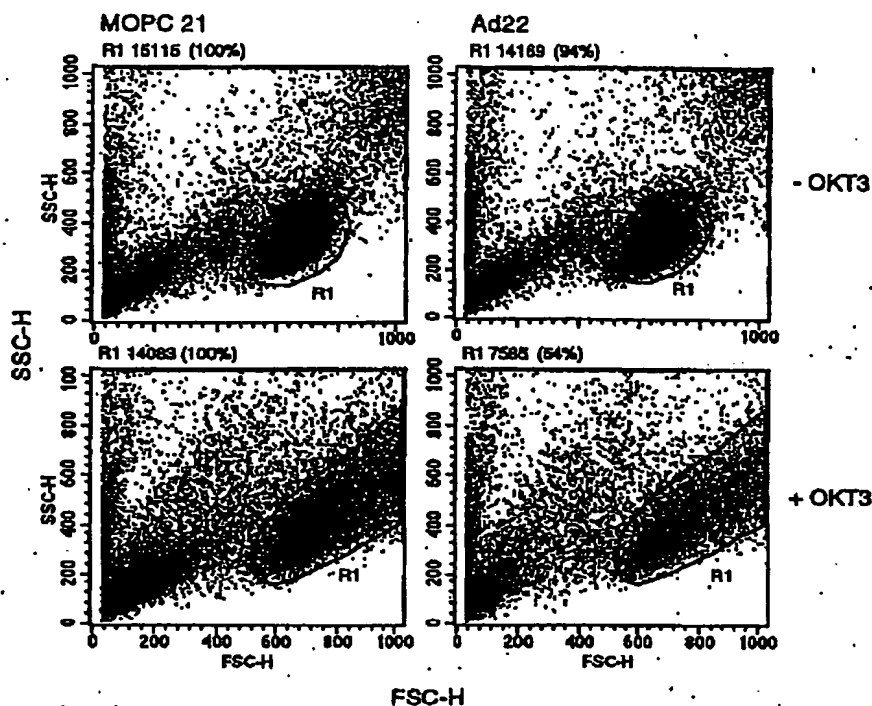
	Percentage of Annexin V Positive Cells ^b					
	1 hour		3 hours		6 hours	
	MOPC-21	Ad22	MOPC-21	Ad22	TEPC 183	CH11
– Inhibitor	8.7 \pm 2.9	49.1 \pm 1.9	9.2 \pm 4.2	52.9 \pm 5.8	7.9 \pm 1.8	28.8 \pm 0.2
+ DMSO (0.5%) ^c	9.9 \pm 1.7	48.5 \pm 4.8	11.9 \pm 4.4	62.5 \pm 3.5	7.8 \pm 0.7	33.6 \pm 2.7
+ Ac-YVAD-CMK	10.2 \pm 1.7	48.1 \pm 1.2	13.5 \pm 1.0	54.6 \pm 1.8	8.8 \pm 1.7	11.5 \pm 2.6
+ Z-VAD-FMK	9.2 \pm 3.4	45.7 \pm 9.5	7.8 \pm 0.01	58.4 \pm 4.9	10.4 \pm 0.4	9.8 \pm 3.3
+ Ac-DEVD-CHO	8.3 \pm 1.6	47.8 \pm 8.6	8.3 \pm 1.8	62.0 \pm 4.0	8.4 \pm 2.8	17.8 \pm 2.7

^a Jurkat E6 cells were preincubated with the indicated ICE/CPP32 inhibitors (100 μ M) for 60 min before further culture with 1 μ g/ml mAb Ad22, CH11, or isotype control as indicated. Cultures with CH11 were included as controls.

^b Annexin V-FLUOS binding was assessed by flow cytometry. Results are expressed as the mean of 10 separate experiments \pm SD.

^c Mock control.

FIGURE 10. mAb Ad22 induces cell death of CD3 ϵ -activated, but not resting, T cells. PBMC (1×10^6 cells/ml) were incubated with Ad22 or isotype control mAb MOPC-21 (2 μ g/ml) as indicated and examined for the effect on cell viability. Region R1 defines living lymphocytes, and the impact of mAb treatment was determined by enumeration of cells in this region using TruCount tubes and flow cytometry. Following incubation, harvested cells were resuspended in an equal volume of buffer and transferred to TruCount tubes containing known numbers of fluorescent microbeads (counts). Under each condition 2000 microbeads were collected, and the number of lymphocytes in region R1 was determined. The numbers above each plot represent the number of cells in region R1 and the percentage of cells in this region compared with that in control culture with MOPC-21. *Upper panel.* Resting PBMC were incubated with mAbs for 24 h and examined for the number of cells in R1. *Lower panel.* PBMC stimulated with the anti-CD3 ϵ mAb OKT3 (1 μ g/ml) for 18 h were finally cultured for an additional 6 h with Ad22 or MOPC-21 and then analyzed.



respond with apoptosis following activation of CD45 (58) and CD99 (49), suggesting a role for these molecules in T cell selection. However, as CD47-deficient mice show normal development of CD4 $^{+}$ and CD8 $^{+}$ T cells (Frederik P. Lindberg, unpublished observations), CD47 has apparently no function in the negative selection of T cells.

CD3, CD45, and the Src kinase p56 lck control early events in TCR signaling. Furthermore, CD4 signaling may also be involved in regulating AICD of T cells (55–57). However, in the context of CD47-mediated apoptosis, we found no requirements for functional CD3, CD4, CD45, or p56 lck . These findings suggest that Ad22-induced apoptosis is not controlled by early signaling events associated with TCR signaling. Interestingly, we also excluded the possibility that CD47-induced apoptosis proceeds by functional engagement of CD45, which recently has been implicated in T and B cell apoptosis (48, 58). Notably, coligation of CD47 and CD3 induced a significant increase in Jurkat T cell apoptosis, as judged by annexin V binding. Thus, coactivation of distinct CD47 epitopes and the TCR/CD3 complex may represent a dramatic challenge to proliferating T cells, resulting in unusual rapid elimination of the cells.

TNF- and FasL-mediated apoptosis are recognized as major pathways in AICD of mature T cells (18–20, 25–27). However, CD47-induced T cell death signaling proceeded independently of TNFR or Fas ligation, apparently by a novel signaling pathway. In this context, programmed cell death may proceed with or without DNA fragmentation. Thus, Fas-mediated apoptosis proceeds with DNA fragmentation into oligonucleosomes (47), whereas CD45, CD99, and HLA class I-mediated apoptosis do not involve observable DNA fragmentation (48, 49, 58, 65, 66). Comparison between Ad22- and CH11-induced apoptosis using the TUNEL assay further demonstrated distinctions between the CD47- and Fas-mediated apoptotic pathways, as Ad22 imposed no characteristic DNA fragmentation even when cells were costimulated with OKT3. An-

other distinct feature of Fas-mediated apoptosis is the requirement for ICE- and CPP32-like proteases (62), which were not needed in CD47-induced T cell death. In contrast, Ad22-induced T cell death revealed close resemblance to the characteristic apoptosis associated with distinct CD45 and HLA class I signaling (48, 65, 66).

Whereas substantial information has recently been achieved on how Fas and TNFR signaling activate the cell's death program, the basis for apoptosis proceeding without caspase or DNA endonuclease activation is not clear, but these novel pathways may represent optional, fast death strategies for efficient clearance of activated cells. Notably, T cells require days to develop susceptibility to Fas and TNFR-mediated cell death (18, 23, 24, 26). Thus, alternative death pathways, as represented by CD45 (48, 58) and CD47, may be required to control FasL- and TNF-insensitive T cells. Furthermore, as some virus may interfere with caspase activation and thereby the induction of conventional programmed cell death (67–69), the immune system may also depend on optional caspase-independent apoptotic pathways to challenge these infections (66). Notably, activation of Fas-independent and non-classical apoptosis has also been implicated in HIV-mediated depletion of CD4 $^{+}$ T cells (70, 71).

In three recent studies purified T cells and immobilized anti-CD3 and anti-CD47 mAbs were used to address the role of CD47 on normal T cells (40–42). The reports demonstrate the ability of CD47 to costimulate T cell activation provided that the mAbs are presented immobilized on the same surface. In contrast, addition of soluble anti-CD47 mAbs had no stimulatory effect even if they were cross-linked. Furthermore, high doses of soluble anti-CD47 mAb 1/1A4 interfered with T cell proliferation after stimulation with suboptimal numbers of dendritic cells (42). However, 1/1A4 did not influence allogeneic MLR induced by optimal numbers of dendritic cells (42). Our studies with Jurkat cells revealed that CD47-mediated death signaling apparently requires an unrestricted

and flexible Ag structure. The impacts of Ad22 and CD47 signaling on normal T cells were addressed using PBMC, as these conditions include cosignaling provided by the normal, hemopoietic cellular environment, including CD28 costimulation. While Ad22 disclosed no impact on unstimulated T cells, preactivation with OKT3 induced a marked T lymphocyte sensitivity to short term Ad22 treatment. Thus, CD47, like Fas and TNFRs, seem to share the feature that expression of the respective Ags is not sufficient to trigger apoptosis, but depend on preactivation to signal cell death. Taken together, the outcome of CD47 engagement on normal T cells is apparently determined by their activation status and the specific CD47 region activated.

CD47 ligands inducing T cell activation or apoptosis have not been identified. However, the Ad22 and 1F7 binding site seems to be exposed at the IgV domain of CD47 near the B6H12 epitopes, suggesting easy access for ligands presented or released by other cells as well as interactions with molecules expressed on the same T cell. Activated T cells induced to express a functional CD47L or encountering CD47L presented on APC would expectedly be efficiently eliminated. Recent reports demonstrate that transformed cells may use a similar strategy for immune escape by expressing FasL (72, 73).

In conclusion, we have found that ligation of distinct CD47 epitopes rapidly induces TNFR- and Fas-independent death of activated T cells. This may indicate that CD47 has a physiological role in clearance of activated T cells by a novel apoptotic pathway. Thus, it will be important to provide further understanding of the involvement of CD47 in the regulation of immune responses.

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